Bacterial and fungal communities associated with Tuber magnatum-productive niches

Bacterial and fungal communities associated with *Tuber magnatum*-productive niches

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Abstract

Truffles are hypogeous ectomycorrhizal fungi of ecological interest for forestry in soils of the northern hemisphere, and of economical relevance for food markets worldwide. The molecular mechanisms that control truffle body formation are largely unknown, as well as the environmental factors that are likely involved. Among the latter, it has been hypothesized that soil-borne communities may have an impact on truffle production. To address this question, we investigated bacterial and fungal communities resident in productive versus adjacent non-productive grounds of the white truffle *Tuber magnatum* by using PCR-DGGE. Although bacterial communities were generally highly similar across all samples within the grounds, profiles did cluster according to the productivity of circumscribed niches, and a *Moraxella osloensis* population appeared to be associated with productive sites. Fungal communities revealed several populations, yet showed no obvious patterns in relation to productivity, although *Mortierella* and *Fusarium oxysporum* appeared to be more abundant in the productive area. Our results offer a first glimpse into microbial communities thriving in truffle productive niches, and open the question as to whether microbe-mediated mechanisms may facilitate/inhibit truffle fruiting-body production or, vice versa, i.e. whether truffle sporocarps have an impact on the microbes living in the rhizosphere.

Keywords: *Moraxella* , *Clavulina* , soil biodiversity, PCR-DGGE, white truffle

Introduction

Truffles are among the few ascomycetous fungi which form ectomycorrhizae with roots of trees, such as *Populus* sp., *Salix* sp., *Quercus* sp., *Tilia* sp., *Pinus* sp. (Smith & Read 1997 Smith, SE and Read, DJ. 1997. “Mycorrhizal symbiosis”. New York: Academic Press. [CrossRef]), and shrubs (Fontana & Giovannetti 1978 Fontana, A and Giovannetti, G. 1978–1979. Simbiosi micorrizica fra *Cistus incanus* L. spp. *incanus* e *Tuber melanosporum* Vitt. *Allionia*, 23: 5–11. –1979). In addition to their ecological interest for forestry, some *Tuber* species have a great economic importance for their organoleptic properties. Among them, specially appreciated is *T. magnatum* Pico, the white truffle commonly known worldwide for its unique taste and flavour. Since truffle fruiting-bodies cannot yet be obtained under controlled conditions, our knowledge of the molecular events leading to ascocarp development is quite limited (Gabella et al. 2005 Gabella, S, Abbà, S, Duplessis, S, Montanini, B, Martin, F and Bonfante, P. 2005. Transcript profiling reveals novel marker genes involved in fruiting body formation in *Tuber borchii*. *Eukaryot Cell*, 4: 1599–1602.). In addition to genetic controls, biotic and abiotic environmental factors are likely involved. Several studies have focused on bacteria (Bedini et al. 1999 Bedini, S, Bagnoli, G, Sbrana, C, Leporini, C, Tola, EDunne, C. 1999. Pseudomonads isolated from within fruitbodies of *Tuber borchii* are capable of producing biological control or phytostimulatory compounds in pure culture. *Symbiosis*, 26: 223–236. ; Sbrana et al. 2000 Sbrana, C, Bagnoli, G, Bedini, S, Filippi, C, Giovanetti, M and Nuti, MP.

2000. Adhesion to hyphal matrix and antifungal activity of *Pseudomonas* strains isolated from *Tuber borchii* ascocarps. *Can J Microbiol*, 46: 259–268. , 2002 Sbrana, C, Agnolucci, M, Bedini, S, Lepera, A, Toffanin, AGiovannetti, M. 2002. Diversity of culturable bacterial populations associated to *Tuber borchii* ectomycorrhizas and their activity on *T. borchii* mycelial growth. *FEMS Microbiol Lett*, 211: 195–201. ; Citterio et al. 2001 Citterio, B, Malatesta, M, Battistelli, S, Marcheggiani, F, Baffone, WSaltarelli, R. 2001. Possible involvement of *Pseudomonas fluorescens* and Bacillaceae in structural modifications of *Tuber borchii* fruitbodies. *Can J Microbiol*, 47: 264–268. ; Barbieri et al. 2007 Barbieri, E, Guidi, C, Bertaux, J, Frey-Klett, P, Garbaye, JCeccaroli, P. 2007. Occurrence and diversity of bacterial communities in *Tuber magnatum* during truffle maturation. *Environ Microbiol*, 9: 2234–2246.) and fungi (Zacchi et al. 2003 Zacchi, L, Vaughan-Martini, A and Angelici, P. 2003. Yeast distribution in a truffle-field ecosystem. *Ann Microbiol*, 53: 275–282.) directly associated with *Tuber* fruiting-bodies, but few data are available about microbial populations thriving in the truffle ecosystem, and their potential role in the truffle life-cycle (Luppi-Mosca 1973 Luppi-Mosca, AM. 1973. La micoflora della rizosfera nelle tartufaie. *Allionia*, 19: 29–32. ; Zacchi et al. 2003 Zacchi, L, Vaughan-Martini, A and Angelici, P. 2003. Yeast distribution in a truffle-field ecosystem. *Ann Microbiol*, 53: 275–282.).

The present study focused on a *T. magnatum* truffle-ground, namely a special natural environment where the precious white truffle is produced. We hypothesized that bacterial and fungal communities would differ inside this area, depending on the specific niches where truffles are produced, and that specific microbial taxa could be related to productive versus non-productive sites. While the appearances of burnt areas in *T. melanosporum* truffle-grounds are signals of future production because environmental conditions are suitable for fruiting (Suz et al. 2008 Suz, LM, Martin, MP, Oliach, D, Fischer, CR and Colinas, CA. 2008. Mycelial abundance and other factors related to truffle productivity in *Tuber melanosporum-Quercus ilex* orchards. *FEMS Microbiol Lett*, 285: 72–78.), productivity in *T. magnatum* truffle-grounds can be exclusively labelled by truffle collection.

With the final aim of identifying microbes as “productivity” markers, we examined the soil microbial communities present in the selected *T. magnatum* truffle-ground in relation to its productive niches, which had been identified upon truffle collection in a long-term survey (Mello et al. 2005 Mello, A, Murat, C, Vizzini, A, Gavazza, V and Bonfante, P. 2005. *Tuber magnatum* Pico, a species of limited geographical distribution: Its genetic diversity inside and outside a truffle-ground. *Environ Microbiol*, 7: 55–65.). Given that ectomycorrhizal fungi interact with soil communities thereby establishing a multitrophic ectomycorrhizal complex (Frey-Klett & Garbaye 2005 Frey-Klett, P and Garbaye, J. 2005. Mycorrhiza helper bacteria: A promising model for the genomic analysis of fungal-bacterial interactions. *New Phytol*, 168: 4–8. ; Frey-Klett et al. 2007 Frey-Klett, P, Garbaye, J and Tarkka, M. 2007. The mycorrhiza helper bacteria revisited. *New Phytol*, 176: 22–36.), a cause–effect relation between the resident microbial communities and the productive niches is envisaged.

PCR-DGGE (Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis) analysis was chosen as the fingerprinting method because of its ability to provide a comprehensive picture of the microbial community structure, and to allow species identification by excising and sequencing individual bands (Muyzer et al. 2004 Muyzer, G, Brinkhoff, T, Nubel, U, Santegoeds, C, Schäfer, H and Wawer, C. 2004. “Denaturing gradient gel electrophoresis (DGGE) in microbial ecology”. In *Molecular microbial ecology manual.* , 2nd ed., Edited by: Kowalchuk, GA, de Bruijn, FJ, Head, IM, Akkermans, ADL and van Elsas, JD. 743–770. Dordrecht: Kluwer Academic.). Bacterial 16S and fungal 18S rRNA genes, amplified from total soil DNA, were analysed by DGGE to produce the profiles of the soil microbial communities in the productive and non-productive areas of the natural truffle-ground. Community similarities were compared, and analyses performed to identify

bands related to productivity status; bands from profiles were identified by sequence analysis where possible.

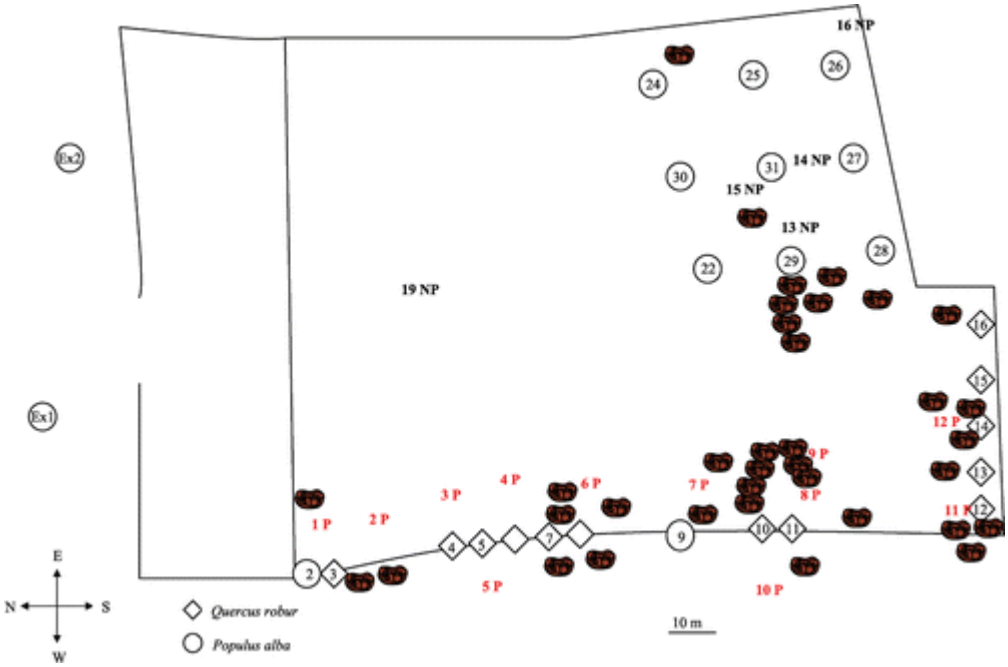
Materials and methods

Study site and sampling

The study site was a truffle-ground of about 7000 m^2 located in a valley in Montemagno (Asti, Piedmont, northern Italy, 8°19′35″4 East- 44°59′2″40 North), where the production dynamics of the truffle have been studied since 1997 (Mello et al. 2005 Mello, A, Murat, C, Vizzini, A, Gavazza, V and Bonfante, P. 2005. *Tuber magnatum* Pico, a species of limited geographical distribution: Its genetic diversity inside and outside a truffle-ground. *Environ Microbiol*, 7: 55–65.).

Seventeen soil samples, about 100 g each, were collected from both the productive (niches: 1P–12P) and non-productive areas (niches: 13NP–16NP, 19NP) of the truffle-ground at about 20–30 cm depth (Figure 1). The limit between productive (P) and non-productive (NP) areas was chosen based on truffle collection sites, and their position. A small area where truffles have never been collected, but located in-between adjacent collection sites (3P–5P), was still considered a productive area. We indicated as non-productive area a wide zone far from the productive one.

Figure 1 Map of the truffle-ground located at Montemagno (AT). Numbers (in red and black) show soil sampling sites in the truffle-ground with respect to the positions of the host trees (circles and diamonds). Areas corresponding to samples 1P–12P (in red) are considered productive based on truffles collected, whereas those corresponding to samples 13NP–16NP and 19NP are considered non-productive.



As reference sites, two soil samples (22NPO, 24NPO) were collected from a different and barely productive truffle-ground situated in Montemagno, 1 km away from the truffle-ground. As control, one soil sample (26) was collected from an orchard, which is a different environment. In all samples pH, soil texture and cationic exchange capacity (Chapman 1965 Chapman, HD. 1965. “Cation-exchange capacity. Methods of soil analysis – Chemical and microbiological properties”. In

Agronomy, Edited by: Black, CA. Vol. 9, 891–901. Madison: American Institute of Agronomy.) were measured before storage at −20°C until further processing.

DNA extraction and PCR

DNA was extracted from soil samples using the FastDNA Spin Kit for Soil (Qbiogene, Heidelberg, Germany) with the modifications described by Luis et al. (2004 Luis, P, Walther, G, Kellner, H, Martin, F and Buscot, F. 2004. Diversity of laccase genes from basidiomycetes in a forest soil. *Soil Biol Biochem*, 36: 1025–1036.). Three replicate samples of 0.5 g of soil were extracted per sample and pooled for analysis.

Polymerase chain reactions (PCRs) were carried out on a TGradient thermocycler (Biometra, Goettingen, Germany) in a final volume of 40 µl containing approximately 5 ng of template DNA, 30 nM of each primer, 400 µM dNTPs, 4 µl 10X buffer and 1 U of Expand High Fidelity DNA polymerase (Roche, Lewews, UK). A negative control (no DNA) was included in each PCR reaction. Amplifications products were electrophoresed in 1.2% (w/v) agarose gels, stained with ethidium bromide and visualised under UV light.

Bacterial 16S rRNA gene fragments were amplified with the primers 968F-GC (AACGCGAAGAACCTTAC + GC-clamp) and 1378R (GGGCGGWGTGTACAAGGC) (Heuer et al. 1997 Heuer, H, Krsek, M, Baker, P, Smalla, K and Wellington, EMH. 1997. Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. *Appl Environ Microbiol*, 63: 3233–3241. 1999 Heuer, H, Harung, K, Wieland, G, Kramer, I and Smalla, K. 1999. Polynucleotide probes that target a hypervariable region of 16S rRNA genes to identify bacterial isolates corresponding to bands of community profiles. *Appl Environ Microbiol*, 65: 1045–1049.), and the following thermocycling programme: 94°C for 2 min, followed by 30 cycles at 92°C for 30 s, 55°C for 1 min and 68°C for 45 s plus 1 s per cycle. The reaction was followed by a final extension at 68°C for 5 min.

Fungal 18S rRNA genes were amplified using the primers FF390 (CGATAACGAACGAGACCT) and FR1-GC (AICCATTCAATCGGTAIT + GC-clamp) (Vainio & Hantula 2000 Vainio, EJ and Hantula, J. 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycol Res*, 104: 927–936.). Cycling parameters were 94°C for 4 min, followed by 2 cycles at 92°C for 30 s, 55°C for 1 min and 68°C for 2 min, 2 cycles at 92°C for 30 s, 53°C for 1 min and 68°C for 2 min, 2 cycles at 92°C for 30 s, 51°C for 1 min and 68°C for 2 min, 2 cycles at 92°C for 30 s, 49°C for 1 min and 68°C for 2 min, and 29 cycles at 92°C for 30 s, 47°C for 1 min and 68°C for 45 s plus 1 s per cycle. The reaction was followed by a final extension at 68°C for 10 min.

PCR-DGGE analysis

For PCR-DGGE analyses 6% (w/v) polyacrylamide gels were prepared. For bacterial community analyses, the linear gradient used was from 44% to 51% in addition to two other gradients, in an attempt to optimise resolution, where 100% denaturing acrylamide is defined as containing 7 M urea and 40% (v/v) formamide (Muyzer et al. 1993 Muyzer, G, De Waal, EC and Uitterlinden, AC. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes for 16S rRNA. *Appl Environ Microbiol*, 55: 695–700.). These analyses yielded highly similar results, with virtually identical profile clustering (results not shown).

For fungal community analyses the linear gradient used was from 35% to 55% denaturant. A 10 ml stacking gel containing no denaturants was added before polymerisation was completed.

All DGGE analyses were carried out in a DCode Universal Mutation Detection System (Bio-Rad, Hemel Hempstead, UK), at a constant temperature of 60°C. Electrophoresis was for 10 min at 180 V, after which the voltage was lowered to 75 V for an additional 16 h.

Gels were stained in 1X TAE containing 0.5 mg l^{-1} ethidium bromide and subsequently washed in 1X TAE prior to be visualised by UV transillumination. Gel images were digitally captured with a VersaDoc Imaging System Model 4000 (Bio-Rad, Hemel Hempstead, UK).

Comparison of DGGE profiles was done, at Heteren, with the ImageMaster programme (Amersham Pharmacia Biotech) as described by Duineveld et al. (2001 Duineveld, BM, Kowalchuk, GA, Keizer, A, Van Elsas, J and Van Veen, JA. 2001. Analysis of the bacterial communities in the rhizosphere of *Chrysanthemum* via denaturing gradient gel electrophoresis of PCR amplified 16S ribosomal RNA and DNA fragments. *Appl Environ Microbiol*, 67: 172–178.) for bacterial communities and, in Torino, with the Quantity One programme (Biorad) for fungal communities. Pearson's index and Dice coefficient were used for bacterial and fungal populations, respectively, taking both band presence and intensity into account after signal normalisation. For dendrogram construction, the obtained similarity matrix was clustered using the UPGMA algorithm (Röling et al. 2000 Röling, WFM, Van Breukelen, BM, Braster, M, Goeltom, MT, Groen, J and Van Verseveld, HW. 2000. Analysis of microbial communities in a landfill leachate polluted acquifer using a new method for anaerobic physiological profiling and 16s rDNA based fingerprinting. *Microb Ecol*, 40: 177–188.).

Peak density and the surface area of each peak from two bands present in PCR-DGGE profiles of all samples were used to determine the abundance of the respective members of the fungal communities. The statistical analyses were performed using $P \leq 0.05$ as the cut-off criterion.

Sequence analysis of DGGE bands

Entire DGGE bands selected for sequence analysis were excised from the UV-illuminated polyacrylamide gels, and DNA was eluted from excised gel fragments by incubation in 30 l of ddH2O at 28°C for 12 h (Ma et al. 2005 Ma, WK, Siciliano, SD and Germida, JJ. 2005. A PCR-DGGE method for detecting arbuscular mycorrhizal fungi in cultivated soils. *Soil Biol Biochem*, 37: 1589–1597.). Eluted DNA was used for PCR amplification as described above. PCR products were analysed again by DGGE, and the products corresponding to DGGE single bands were purified for sequence analysis using the QiaQuick PCR purification kit (Qiagen, Hilden, Germany) with a final elution of 30 ml. Sequencing reactions, using the bacterial primer 1378 and the fungal primer FF390, were performed by the Dinamycode Laboratories (Torino, Italy).

Sequence similarity was analysed using the BLAST algorithm (Basic Local Alignment Search Tools) available on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The accession number for the band sequenced from the bacterial community is FM202465, whereas those for fungi are listed in Table I.

Table I. Blast results from band sequences and accession numbers. The first Blast hits (1–4) and affiliation deduced (in bold) are given.

Band number	Accession number	Blast results	E value
1b	FM202457	– Uncultured fungus– *Sebacina* sp.Basidiomycota, Agaricomycotina, Agaricomycetes	2e-1479e-1479e-147
2	FM202449	– Uncultured Pezizaceae– Uncultured PezizaceaeAscomycota, Pezizomycotina, Pezizomycetes, Pezizales, Pezizaceae	1e-1571e-157
3b	FM202450	– Uncultured fungus– *Tricholoma matsutake*– Uncultured EukaryoteBasidiomycota, Agaricomycotina, Agaricomycetes, Agaricales, Tricholomataceae	5e-1565e-1556e-155
3c	FM202451	– Uncultured Eukaryote– Uncultured Eukaryote– Uncultured Eukaryote	2e-1552e-1552e-155
4a	FM202458	– *Metarhizium anisopliae*– *Metarhizium anisopliae*Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreales, Clavicipitaceae	4e-1574e-157
4b	FM202459	– *Inocybe* sp.– Uncultured soil fungus– *Tricholomella* sp.– *Xeromphalina* sp.Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricales	2e-1612e-1614e-1594e-159
6	FM202460	– *Inocybe* sp.– Uncultured soil fungus– *Tricholomella* sp.Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricales	3e-1603e-1606e-158
7a, 10a	FM202452	– *Fusarium* sp.– *Fusarium oxysporum*Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreales, Hypocreaceae, *Fusarium oxysporum*	3e-1463e-146
8	FM202461	– Uncultured fungus– *Pleurotellammula* sp.– *Tricholoma* sp.Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricales	3e-1604e-1594e-159
9, 11	FM202462	– Uncultured fungus– *Mortierella chlamydospora*– *Mortierella alpina*Zygomycota, Mucoromycetes, Mortierellales, Mortierellaceae, *Mortierella*	8e-1732e-1672e-167
12	FM202463	– Uncultured fungus– *Tricholomella* sp.– *Xeromphalina* sp.Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricales, Tricholomataceae	8e-1703e-1663e-166
10b, 13, 14, 15	FM202464	– *Clavulina* sp.– *Clavulina cristata*– *Hydnum* sp.Basidiomycota, Agaricomycotina, Agaricomycetes, Cantharellales, Clavulinaceae, *Clavulina cristata*	3e-1603e-1606e-152
15c	FM202453	– *Psilocybe stuntzii*– *Psilocybe silvatica*Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricales, Strophariaceae, *Psilocybe*	5e-1565e-156
16c	FM202454	– Uncultured fungus– *Cortinarius sodagnitus*– *Cortinarius violaceus*Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricales, Cortinariaceae	6e-1557e-1547e-154
19	FM202455	– *Pleurotopsis longinqua*– *Tricholomella constricta*Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricales, Tricholomataceae	1e-1571e-157
22c	FM202456	– *Cortinarius sodagnitus*– *Cortinarius violaceus*Basidiomycota, Agaricomycotina, Agaricomycetes, Agaricales, Cortinariaceae, *Cortinarius*	2e-1602e-160

Results

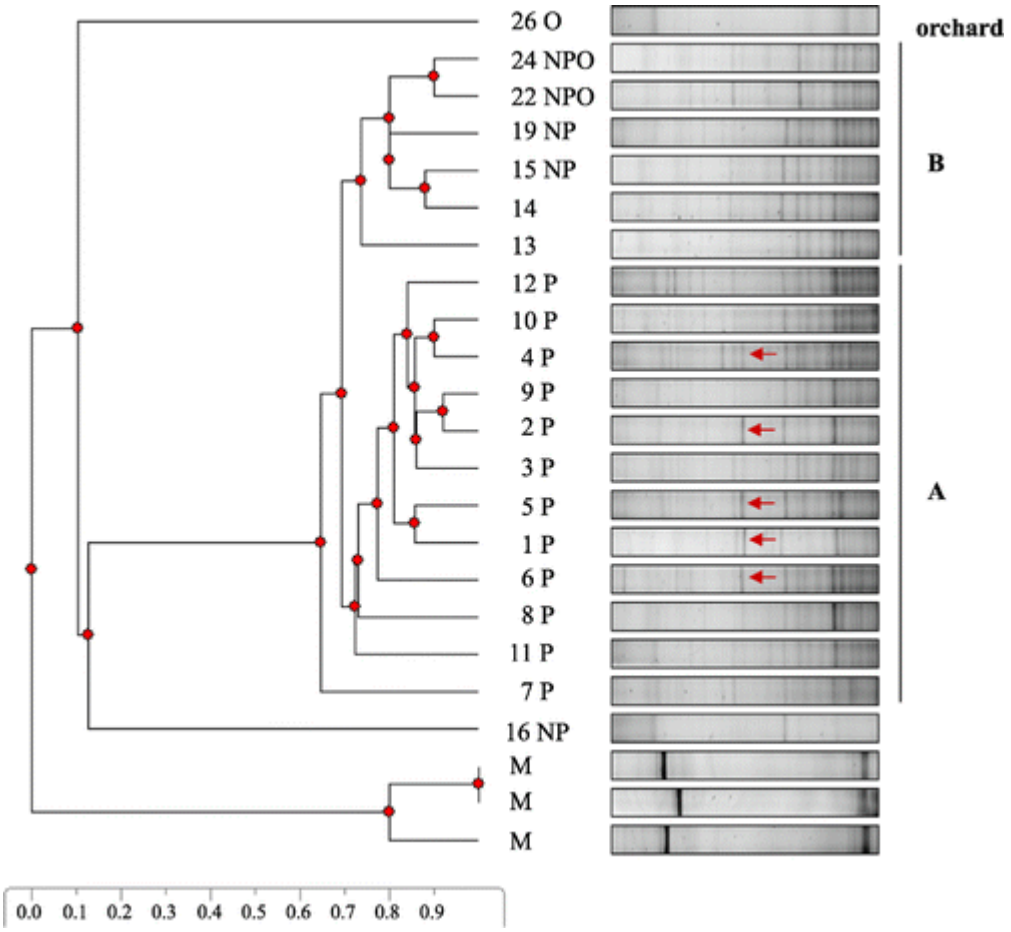
DGGE profiles of bacterial 16S fragments

PCR amplification of the bacterial 16S rDNA region with the primer couple 968FGC and 1378 resulted in an intense amplification product of approximately 400 bp. In all three considered gradients, DGGE analysis of the PCR products generated a complex banding pattern for each of the collected soil samples, to an extent that profiles showed numerous faint unresolved bands consistent with the very large bacterial diversity typical of soil environments (Schloss & Handelsman 2006 Schloss, PD and Handelsman, J. 2006. Toward a census of bacteria in soil. *PLoS Comput Biol*, 2: 786–793.).

DGGE profiles were reproducible for all samples (data not shown), indicating that the variation in DGGE patterns is representative for differences among soil samples. The profiles from two different DGGE runs were compared, in two independent analyses, to determine similarities.

The analysis of banding patterns obtained with the three gradients gave nearly identical results: the presence of two large clusters, with about 70% similarity score (Figure 2). One cluster (A) mostly comprised soil samples collected in the productive area, while the other cluster (B) included soil samples coming from the non-productive area, as well as from the poorly productive truffle-ground sampled as a reference. Sample 16NP, at the border of the truffle-ground, did not cluster with any sample, nor did the soil coming from the orchard site.

Figure 2 Dendrogram resulting from the image analysis of 16S rDNA profiles. Samples from the productive area cluster in Group A, whereas most samples from the non-productive area cluster in Group B.



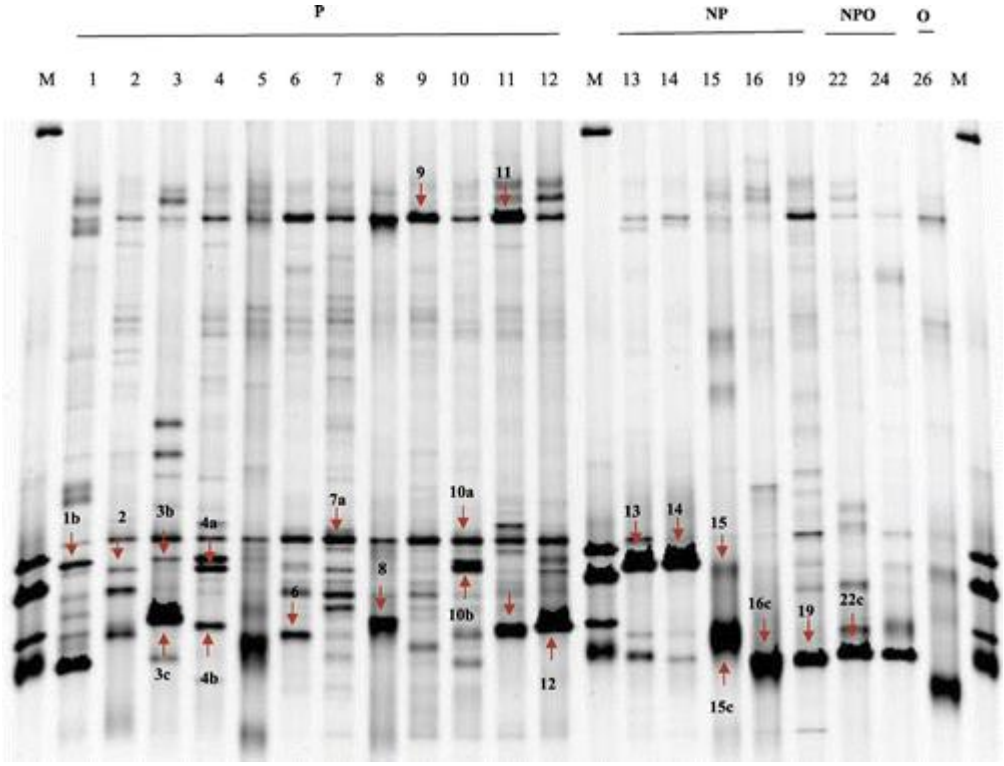
In order to understand whether bacterial profile clusters were more related to truffle presence than to soil characteristics, these were compared between P and NP areas. Clear differences were not revealed, since pH was 7.5 in all the niches, soil texture was homogenously silty clay loam, and cationic exchange capacity evaluated as high (about 50 meq/100 g).

The faint nature of most bands in bacterial PCR-DGGE profiles hampered our ability to retrieve clean sequence information for excised bands, with the exception of a single defined band (see Figure 2) in many samples (1P, 2P, 4P, 5P, 6P) from the productive area. The sequence of this band was determined from samples 1P and 6P. The same sequence was obtained from the two samples, as expected, revealing 100% identity with *Moraxella osloensis* Bøvre & Henriksen 16 rRNA gene sequence, a bacterium belonging to the *gamma*-Proteobacteria group.

DGGE profiles of fungal 18S rRNA gene fragments

As with bacterial community profiles, fungal profiles showed a high level of reproducibility in three different runs (data not shown). Fungal-specific PCR-DGGE profiles revealed high levels of spatial heterogeneity across the study site (Figure 3). DGGE profiles from two experiments were compared, in two independent analyses, to determine profile similarities and examine any clustering of samples according to fungal community structure. Samples did not cluster with respect to truffle production, and unclear bands could be identified that corresponded to either the productive or non-productive samples (data not shown).

Figure 3 DGGE separation patterns of PCR-amplified 18S rDNA fragments from soil samples. Productive (P) and non-productive (NP) areas of the investigated truffle-ground, outer non-productive (NPO) areas, and orchard (O) samples are indicated.



In order to investigate the fungal diversity inside the truffle-ground, 21 bands (identified by arrows in Figure 3), from the most dominant fungal populations, were excised and sequenced. Some bands corresponding to the same gel electrophoresis lane were excised and sequenced in order to verify the correctness of the image analysis. A total of 16 different sequences were obtained, and the blast sequence similarities are shown in Table I where the first blast hits (1–4) are provided. Given that reliability of published sequences has been debated (Bridge et al. 2004 Bridge, PD, Spooner, BM and Roberts, PJ. 2004. Reliability and use of published sequence data. *New Phytol*, 161: 1–21. ; Nilsson et al. 2006 Nilsson, RH, Ryberg, M, Kristiansson, E, Abarenkov, K, Larsson, KH and Kõljalg, U. 2006. Taxonomic reliability of DNA sequences in public sequence databases: A fungal perspective. *PLoS ONE*, 1: e59 doi:10.1371/journal.pone.0000059), the blast hits are followed by our interpretation of the database on the basis of current knowledge on fungal systematics. The recovered sequences presented similarity with 11 sequences of Basidiomycota, 3 sequences of Ascomycota and 1 of Zygomycota. In only six cases the recovered sequences show a similarity with known taxa (species and genera) – *Fusarium oxysporum* Schltdl., *Clavulina cristata* (Holmsk.) J. Schröt. [current name *Clavulina coralloides* (L.) J. Schröt.], *Metarhizium anisopliae* (Metschn.) Sorokin, *Cortinarius*, *Psilocybe*, *Mortierella*. In all the remaining cases, the band sequences could be ascribed to orders and/or families. The band (9, 11 in Table I and Figure 3) corresponding to *Mortierella* was present in all samples, and was very intense in most samples from the productive niches. An analysis of the band density (reflecting dominance) showed a significant (*t*-test, $P < 0.05$) abundance of *Mortierella* in the productive area in comparison to that in the corresponding non-productive area (p-value = 0.4×10^{-2}). Similarly, the band (7a, 10a) corresponding to *F. oxysporum* was present in all the samples from the productive niches with a strong signal, while it was faintly detectable in the samples from the non-productive niches. Analysis of its density showed a significant (*t*-test, $P < 0.05$) abundance of *F. oxysporum* in the productive niches in comparison to that in the corresponding non-productive niches (p-value = 0.2×10^{-3}).

Discussion

Our knowledge of the molecular events causing ectomycorrhizal fungi to develop fruiting-bodies is quite limited: the transition from the symbiotic mycorrhizal status towards mushroom production is likely dependent on genetic traits and environmental factors (Martin et al. 2007 Martin, F, Kohler, A and Duplessis, S. 2007. Living in harmony in the wood underground: Ectomycorrhizal genomics. *Curr Opin Plant Biol*, 10: 204–210.). Truffle fruiting-bodies cannot yet be obtained under controlled conditions preventing us to follow, step by step, their morphogenetic events (Gabella et al. 2005 Gabella, S, Abbà, S, Duplessis, S, Montanini, B, Martin, F and Bonfante, P. 2005. Transcript profiling reveals novel marker genes involved in fruiting body formation in *Tuber borchii*. *Eukaryot Cell*, 4: 1599–1602.). In the framework of a long-term project aimed at identifying the ecological scenario which leads to truffle production, we wondered whether microbial populations have an impact on truffle life-cycle, for example through the release of rhizospheric signals, which may activate complex gene networks, as described in Deveau et al. (2007 Deveau, A, Palin, B, Delaruelle, C, Peter, M, Kohler, APierrat, JC. 2007. The mycorrhiza helper *Pseudomonas fluorescens* Bbc6R8 has a specific priming effect on the growth, morphology and gene expression of the ectomycorrhizal fungus *Laccaria bicolor* S238N. *New Phytol*, 175: 743–755.).

Here we demonstrate that bacterial-specific community profiles showed high degrees of similarity across all samples in our study area. Despite this, two groups could be discerned in comparative analyses, one corresponding to all samples from the productive niches (Cluster A) and one to most non-productive and nearby low productivity sites (Cluster B). Since the productive areas encompass samples close to each other, the two DGGE profiles could simply reflect sample distances in the field. On the other hand, Cluster B does not group all nearby samples from the non-productive niches (i.e. 16NP), while it comprises also samples which were situated 1 km away from the investigated truffle-ground. In addition, soil characteristics of both P and NP areas resulted to be similar. Notwithstanding the heterogeneity of microbial distribution in the soil (Zhou et al. 2004 Zhou, J, Xia, B, Huang, H, Palumbo, AV and Tiedje, JM. 2004. Microbial diversity and heterogeneity in sandy subsurface soils. *Appl Environ Microbiol*, 70: 1723–1734. from these considerations we can assume that the two bacterial profile clusters are more related to the truffle presence than to sample distances and soil characteristics.

The most distinctive band associated with productive sites showed a high degree of similarity with the *gamma*-Proteobacterium *M. osloensis*. The relation between this bacterial group and productive truffle sites is in line with previous results, based on bacterial cultivation from *T. borchii* fruit-bodies, and detection of various *gamma*-Proteobacteria (Barbieri et al. 2005 Barbieri, E, Bertini, L, Rossi, I, Ceccaroli, P, Saltarelli, RGuidi, C. 2005. New evidence for bacterial diversity in the ascoma of the ectomycorrhizal fungus *Tuber borchii*. *FEMS Microbiol Lett*, 247: 23–35.). In 1995, Citterio et al. isolated bacteria belonging to *Micrococcus*, *Moraxella*, *Pseudomonas* and *Staphylococcus* from *T. magnatum*, *T. maculatum* and *T. borchii* ascomata. Fluorescent pseudomonads were assumed to be able to affect ascospore germination within fruit-bodies of *T. borchii* facilitating ascus opening (Gazzanelli et al. 1999 Gazzanelli, G, Malatesta, M, Pianetti, A, Baffone, W, Stocchi, V and Citterio, B. 1999. Bacteria associated to fruit bodies of the ecto-mycorrhizal fungus *Tuber borchii* Vittad. *Symbiosis*, 26: 211–219.). Recently, Barbieri et al. (2007 Barbieri, E, Guidi, C, Bertaux, J, Frey-Klett, P, Garbaye, JCeccaroli, P. 2007. Occurrence and diversity of bacterial communities in *Tuber magnatum* during truffle maturation. *Environ Microbiol*, 9: 2234–2246.) identified many of the *T. magnatum*-associated bacteria as *gamma*-Proteobacteria, and although the majority was similar to fluorescent pseudomonads, several were affiliated with the Moraxellaceae. Interestingly, *M. osloensis* is associated with *Phasmarhabditis hermaphrodita*, a parasitic nematode that is lethal to slugs, especially the gray garden slug

Deroceras reticulatum, through a lipopolysaccharide, an endotoxin(s) produced by this bacterium (Tan & Grewal 2003 Tan, L and Grewal, PS. 2003. Characterization of the first molluscicidal lipopolysaccharide from *Moraxella osloensis*. *Appl Environ Microbiol*, 69: 3646–3649.). Its effect in the biocontrol of slugs suggests an active role in the complex environment where soil, viruses, archaea, eubacteria, protozoa, algae, plants, insects, nematodes and fungi interact.

It should be noted that our failure to detect the *Moraxella*-affiliated population in non-productive sites does not demonstrate its absence in these sites, but that it might be below the level of detection of the PCR-DGGE approach used (estimated at approximately 1% of total community; Muyzer 1999 Muyzer, G. 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr Opin Microbiol*, 2: 317–322.). Nevertheless, *M. osloensis* seems to be a promising marker of truffle presence or productivity. Whether the presence of this bacterium is the cause or effect of the productivity cannot be understood in field conditions.

In contrast to bacterial communities, fungal profiles did not reveal any clustering with respect to the productivity of the sampled niches: no specific fungal population could be associated with truffle production sites, at the reached detection level. Due to the sharp and distinctive band profiles, the most common fungal populations were successfully identified. Basidiomycota are the most represented phylum: Agaricales with nine sequences and three families (Tricholomataceae, Strophariaceae and Cortinariaceae were the most represented order (Table I). Among the Basidiomycota, Ascomycota and Zygomycota, only three sequences were closely related to known species, allowing their identification at the genus level (*Cortinarius*, *Psilocybe*, *Mortierella)*. The ectomycorrhizal *Cortinarius* is the largest mushroom genus in the world, containing an astounding number of species (ca. 1500 species described): here, it was found in the sample 22NPO.

Psilocybe, found in sample 15NP, is a saprotrophic small mushroom with a worldwide distribution, and well known for the hallucinogenic properties of some species. The zygomycete genus *Mortierella* encompasses saprotrophic soil organisms, which are present all over the truffle-ground. The results are in accord with those of Luppi-Mosca (1973 Luppi-Mosca, AM. 1973. La micoflora della rizosfera nelle tartufaie. *Allionia*, 19: 29–32.), who found *Mortierella alpina* to be common in truffle soils via a traditional cultivation approach method. Murat et al. (2005 Murat, C, Vizzini, A, Bonfante, P and Mello, A. 2005. Morphological and molecular typing of the below-ground fungal community in a natural *Tuber magnatum* truffle-ground. *FEMS Microbiol Lett*, 245: 307–313.) were also able to detect this species, using molecular tools, on mycorrhizal samples collected in the same truffle-ground examined in our study. In addition to this we found that a *Mortierella* sp. is significantly abundant in the productive area. On the whole, present results open new perspectives on the relations occurring among soil micro-organisms, and indicate *Mortierella* as a candidate genus for further studies.

Another three sequences were identical to databank entries, allowing their identification to the species level: *F. oxysporum*, *M. anisopliae and C. cristata. F. oxysporum* is a widespread cosmopolitan phytophatogenic fungus, which can be the causative agent of vascular wilt diseases (Smith et al. 1988 Smith, IM, Dunez, J, Phillips, DH, Lelliott, RA and Archer, SA. 1988. “European handbook of plant diseases”. Oxford: Blackwell Scientific. [CrossRef]). It was abundant in the productive area, as reported above for *Mortierella*. *M. anisopliae* was found in sample 4P. It is an entomopathogenic fungus that grows naturally in soils worldwide and can cause diseases in various insects, hence its use as a biocontrol agent (Butt 2002 Butt, TM. 2002. “Use of entomogenous fungi for the control of insect pests”. In *The Mycota*, Edited by: Esser, K and Bennett, JW. 111–134. Berlin: Springer-Verlag.). *C. cristata* was particularly abundant in the niches – 13NP and 14NP – of the non-productive area. Its fruiting-bodies had already been collected in the truffle-ground in previous investigations (Vizzini, personal communication) suggesting a potential role as guild-

fungus based on recently published information on fungi belonging to the genus *Clavulina*. This genus, recently ascribed to the ectomycorrhizal fungi, has gained increasing attention in the recent years as a relevant component of ectomycorrhizal communities. A study dealing with this genus showed a non-random distribution of *C. cinerea* in ectomycorrhizae and forest floor samples harvested in a red pine forest in Pennsylvania (Koide et al. 2005 Koide, RT, Xu, B, Sharda, J, Lekberg, Y and Ostiguy, N. 2005. Evidence of species interactions within an ectomycorrhizal fungal community. *New Phytol*, 165: 305–316.). In particular, negative associations involving *C. cinerea* or *Cenococcum geophilum*, species that seem to be mutually exclusive, were observed, suggesting that interactions among species can structure communities. In the same study, *C. cinerea* resulted the second more abundant species in soils. Interestingly, *C. cristata* mycorrhizae were also found in an artic-alpine area of Swedish Lapland associated with prostrate willows (*Salix herbacea* and *Salix polaris*) (Clemmensen & Michelsen 2006 Clemmensen, KE and Michelsen, A. 2006. Integrated long-term responses of an arctic-alpine willow and associated ectomycorrhizal fungi to an altered environment. *Can J Bot-Rev Can Bot*, 84: 831–843.), while *C. cinerea* mycorrhizae dominated an Italian forest of Quercus robur (Mosca et al. 2007 Mosca, E, Montecchio, L, Sella, L and Garbaye, J. 2007. Short-term effect of removing tree competition on the ectomycorrhizal status of a declining pedunculate oak forest (*Quercus robur* L.). *For Ecol Manage*, 244: 129–140. [CrossRef], [Web of Science ®]) and a forest of *Picea abies* in Russia (Ivanov 2005 Ivanov, DM. 2005. Mycobionts of ectomycorrhizal rootlets of *Picea abies* in bilberry spruce forest (Leningrad Region). *Mikologiya I Fitopatologiya*, 39: 41–47.). *Clavulina* sp. was also found in a forest of *Pinus sylvestris* in The Netherlands (Landeweert et al. 2005 Landeweert, R, Leeflang, P, Smit, E and Kuyper, TW. 2005. Diversity of an ectomycorrhizal fungal community studied by a root tip and total soil DNA approach. *Mycorrhiza*, 15: 1–6.).

Altogether, the results suggest the need for more detailed studies on the effect of this ubiquitous fungal genus.

Although predictable bias is envisaged in the choice of the differently productive areas (productivity in *T. magnatum* truffle-grounds can be exclusively labelled by truffle collection), this work shows that some micro-organisms are related to the presence of *T. magnatum* fruiting-bodies. Whether or not these are able to structure communities, or whether the bacterial–fungal interactions are able to promote truffle growth is an open question.

Quantitative assays in the soil are necessary to analyse thoroughly these populations, as well as microcosm experiments to directly demonstrate the relations observed in this study. In conclusion, our work describes, for the first time, the soil microbial communities inhabiting a natural truffle-ground. We observed a potential relationship between the presence of truffle fruiting-bodies and the *gamma*-Proteobacterium *M. osloensis*. Moreover, this research offers a first glimpse on the most represented soil fungal populations in these soils and reveals a relationship between the productive area and the abundance of a *Mortierella* sp. and *F. oxysporum*. Lastly, this investigation has yielded data on the presence of the ectomycorrhizal genus *Clavulina* in these fungal communities.

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